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Neue für das luxR-Gen kodierende Nukleotidsequenzen

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Munich, 13th June 2001

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Faust

Novel nucleotide sequences coding for the luxR gene

The invention provides nucleotide sequences from coryneform bacteria coding for the luxR gene, and a process for the production, by fermentation, of amino acids, particularly
5 L-lysine, by attenuating the luxR gene. The luxR gene codes for the LuxR protein, which is a transcription activator.

Prior art

L-amino acids, particularly L-lysine, are used in human medicine and in the pharmaceuticals industry, in the food-
10 stuffs industry and, most particularly, in the feeding of animals.

It is known that amino acids are produced by fermentation of strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great
15 importance, attempts are continuously being made to improve the production processes. Improvements to the processes may concern measures relating to the fermentation, such as, for example, stirring and oxygen supply, or the composition of the nutrient media, such as, for example, the sugar
20 concentration during the fermentation, or working up to the product form by, for example, ion-exchange chromatography, or the intrinsic performance properties of the microorganism itself.

In order to improve the performance properties of such
25 microorganisms, methods of mutagenesis, selection and mutant selection are employed. Such methods yield strains which are resistant to antimetabolites or are auxotrophic for metabolites that are important in terms of regulation, and which produce amino acids.

30 For a number of years, methods of recombinant DNA technology have also been used for improving the strain of L-amino acid-producing strains of *Corynebacterium*.

Object of the invention

The inventors have set themselves the object of providing novel measures for the improved production of amino acids, in particular L-lysine, by fermentation.

5 Description of the invention

The invention provides an isolated polynucleotide from coryneform bacteria, containing a polynucleotide sequence coding for the luxR gene, selected from the group

- 10 a) polynucleotide that is at least 70% identical with a polynucleotide that codes for a polypeptide containing the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide that codes for a polypeptide containing an amino acid sequence that is at least 70% identical with the amino acid sequence of SEQ ID No. 2,
- 15 c) polynucleotide that is complementary to the polynucleotide of a) or b), and
- d) polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c),
- 20 the polypeptide preferably exhibiting the activity of the transcription activator LuxR.

The invention also provides the above-mentioned polynucleotide, it preferably being a replicatable DNA containing:

- 25 (i) the nucleotide sequence shown in SEQ ID No. 1, or
- (ii) at least one sequence that corresponds to sequence (i) within the region of the degeneracy of the genetic code, or

- (iii) at least one sequence that hybridizes with the sequences that are complementary to sequence (i) or (ii), and optionally
- (iv) sense mutations in (i) that are neutral in terms of function.
- 5

The invention also provides:

- a replicatable DNA containing the nucleotide sequence as shown in SEQ ID No. 1;
- a polynucleotide that codes for a polypeptide containing the amino acid sequence as shown in SEQ ID No. 2;
- 10
- a vector containing the polynucleotide according to the invention, point d, particularly pCR2.1luxRint, deposited in Escherichia coli DSM 13619 at the DSMZ, Braunschweig (Germany);
- 15 and coryneform bacteria that contain in the luxR gene an insertion or deletion, particularly using the vector pCR2.1luxRint.

The invention also provides polynucleotides consisting substantially of a polynucleotide sequence, which are obtainable by screening, by means of hybridization, a corresponding gene library that contains the complete gene having the polynucleotide sequence according to SEQ ID No. 1, using a probe containing the sequence of the mentioned polynucleotide according to SEQ ID No. 1 or a fragment thereof, and isolating the mentioned DNA sequence.

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Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate in their complete length nucleic acids or polynucleotides or genes that code for the LuxR protein, or in order to isolate nucleic acids or polynucleotides or

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genes that have a high degree of similarity with the sequence of the luxR gene.

Polynucleotide sequences according to the invention are also suitable as primers, with the aid of which it is possible, by means of the polymerase chain reaction (PCR), to produce DNA of genes that code for the LuxR protein.

Such oligonucleotides acting as probes or primers contain at least 30, preferably at least 20, very particularly preferably at least 15, consecutive nucleotides. Also suitable are oligonucleotides having a length of at least 40 or 50 nucleotides.

"Isolated" means removed from its natural environment.

"Polynucleotide" generally refers to polyribonucleotides and polydeoxyribonucleotides, it being possible for the RNA or DNA to be unmodified or modified.

"Polypeptides" are to be understood as being peptides or proteins that contain two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, particularly those having the biological activity of the LuxR protein, and also those that are at least 70%, preferably at least 80% and in particular at least from 90% to 95% identical with the polypeptide according to SEQ ID No. 2, and exhibit the mentioned activity.

The invention also provides a process for the production of amino acids, particularly L-lysine, by fermentation using coryneform bacteria which, in particular, already produce amino acids and in which the nucleotide sequences coding for the luxR gene are attenuated, in particular excluded or expressed at a low level.

The term "attenuation" in this connection describes the diminution or exclusion of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded for by the corresponding DNA, by, for example, using
5 a weak promoter or using a gene or allele that codes for a corresponding enzyme having low activity, or by inactivating the corresponding gene or enzyme (protein), and optionally by combining those measures.

The microorganisms provided by the present invention are
10 able to produce amino acids, in particular L-lysine, from glucose, saccharose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They may be representatives of coryneform bacteria, particularly of the genus *Corynebacterium*. In the case of the genus
15 *Corynebacterium*, special mention may be made of the species *Corynebacterium glutamicum*, which is known to those skilled in the art for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, particularly of the species *Corynebacterium glutamicum* (*C. glutamicum*),
20 are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
25 *Corynebacterium thermoaminogenes* FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

or L-amino acid-producing mutants or strains prepared
30 therefrom, such as, for example, the L-lysine-producing strains

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708

- Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum DM58-1
5 Corynebacterium glutamicum DG52-5
Corynebacterium glutamicum DSM 5714 and
Corynebacterium glutamicum DSM 12866.

The inventors have succeeded in isolating the new luxR gene of *C. glutamicum* that codes for the LuxR protein, which
10 gene is a transcription activator.

In order to isolate the luxR gene or also other genes of *C. glutamicum*, a gene library of that microorganism in *Escherichia coli* (*E. coli*) is first prepared. The preparation of gene libraries is written down in generally
15 known textbooks and handbooks. There may be mentioned as an example the textbook of Winnacker: *Gene und Klone, Eine Einführung in die Gentechnologie* (Verlag Chemie, Weinheim, Germany, 1990) or the handbook of Sambrook et al.: *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor
20 Laboratory Press, 1989). A very well known gene library is that of the *E. coli* K-12 strain W3110, which has been prepared by Kohara et al. (*Cell* 50, 495-508 (1987)) in λ -vectors. Bathe et al. (*Molecular and General Genetics*, 252:255-265, 1996) describe a gene library of *C. glutamicum*
25 ATCC13032, which has been prepared with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, *Proceedings of the National Academy of Sciences USA*, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, *Nucleic Acids Research* 16:1563-1575). Börmann et al. (*Molecular*
30 *Microbiology* 6(3), 317-326 (1992)) in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pH79 (Hohn and Collins, 1980, *Gene* 11, 291-298).

For the preparation of a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322
35 (Bolivar, 1979, *Life Sciences*, 25, 807-818) or pUC9 (Vieira

et al., 1982, Gene, 19:259-268). Suitable hosts are particularly those E. coli strains that are restriction- and recombination-defective, such as, for example, the strain DH5 α (Jeffrey H. Miller: "A Short Course in
5 Bacterial Genetics, A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria", Cold Spring Harbour [sic] Laboratory Press, 1992).

The long DNA fragments cloned with the aid of cosmids or other λ -vectors can then in turn be subcloned into
10 customary vectors suitable for DNA sequencing.

Methods of DNA sequencing are described *inter alia* in Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America USA, 74:5463-5467, 1977).

15 The resulting DNA sequences can then be studied using known algorithms or sequence-analysis programs, such as, for example, that of Staden (Nucleic Acids Research 14, 217-232 (1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of
20 Biochemical Analysis 39, 74-97 (1998)).

In that manner, the novel DNA sequence of C. glutamicum coding for the luxR gene has been obtained, which sequence, as SEQ ID No. 1, forms part of the present invention.
Furthermore, the amino acid sequence of the corresponding
25 protein has been derived from the present DNA sequence using the methods described above. The resulting amino acid sequence of the luxR gene product is shown in SEQ ID No. 2.

Coding DNA sequences that result from SEQ ID No. 1 by the degeneracy of the genetic code also form part of the
30 invention. Likewise, DNA sequences that hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 form part of the invention. Furthermore, to those skilled in the art, conservative amino acid substitutions, such as, for

example, the substitution of glycine with alanine or of aspartic acid with glutamic acid, in proteins are known as sense mutations, which do not lead to any fundamental change in the activity of the protein, that is to say are
5 neutral in terms of function. It is also known that changes at the N- and/or C-terminus of a protein do not substantially impair its function or may even stabilise it. The person skilled in the art will find relevant information *inter alia* in Ben-Bassat et al. (Journal of
10 Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences that result in a
15 corresponding manner from SEQ ID No. 2 likewise form part of the invention.

Finally, DNA sequences that are produced by the polymerase chain reaction (PCR) using primers that result from SEQ ID No. 1 form part of the invention. Such oligonucleotides
20 typically have a length of at least 15 nucleotides.

The person skilled in the art will find instructions on the identification of DNA sequences by means of hybridization *inter alia* in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH
25 (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). The person skilled in the art will find instructions on the amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) *inter alia* in the hand-
30 book of Gait: Oligonukleotide [sic] Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

During work on the present invention it has been found that
35 coryneform bacteria produce amino acids, in particular

L-lysine, in an improved manner after attenuation of the luxR gene.

In order to achieve attenuation, either the expression of the luxR gene or the catalytic properties of the enzyme
5 protein can be diminished or excluded. The two measures may optionally be combined.

A diminution of gene expression can be effected by carrying out the culturing in a suitable manner or by genetic alteration (mutation) of the signal structures of gene
10 expression. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome-binding sites, the start codon and terminators. The person skilled in the art will find information thereon, for example, in patent
15 application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Pátek et al. (Microbiology 142: 1297 (1996)), Vasicova
20 et al. (Journal of Bacteriology 181: 6188 (1999)) and in known textbooks of genetics and molecular biology, such as, for example, the textbook of Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that of Winnacker ("Gene und Klone", VCH
25 Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change in or diminution of the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the works of Qiu and Goodman (Journal of Biological Chemistry 272:
30 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Reports from the Jülich Research
35 Centre, Jül-2906, ISSN09442952, Jülich, Germany 1994).

Summaries are to be found in known textbooks of genetics and molecular biology, such as, for example, that of Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

5 There come into consideration as mutations transitions, transversions, insertions and deletions. In dependence on the effect of the amino acid substitution on the enzyme activity, missense mutations or nonsense mutations are referred to. Insertions or deletions of at least one base
10 pair (bp) in a gene lead to frame shift mutations, as a result of which incorrect amino acids are incorporated or the translation breaks off prematurely. Deletions of several codons typically result in a complete loss of enzyme activity. Instructions for the production of such
15 mutations are part of the prior art and are to be found in known textbooks of genetics and molecular biology, such as, for example, the textbook of Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that of Winnacker ("Gene und Klone", VCH
20 Verlagsgesellschaft, Weinheim, Germany, 1990) or that of Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

A common method of mutating genes of *C. glutamicum* is the method of gene disruption and of gene replacement described
25 by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

In the method of gene disruption, a central portion of the coding region of the gene in question is cloned into a plasmid vector which is able to replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Suitable
30 vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-5465 (1992)), pGEM-T (Promega corporation, Madison,
35 WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological

Chemistry 269:32678-32684; US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Netherlands; Bernard *et al.*, Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf *et al.*, 1991, Journal of Bacteriology 173:4510-5 4516). The plasmid vector containing the central portion of the coding region of the gene is then transferred to the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, in Schäfer *et al.* (Applied and Environmental 10 Microbiology 60, 756-759 (1994)). Methods of transformation are described, for example, in Thierbach *et al.* (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch *et al.* (FEMS Microbiological Letters 123, 343-347 (1994)). 15 After homologous recombination by means of a cross-over occurrence, the coding region of the gene in question is disrupted by the vector sequence, and two incomplete alleles lacking the 3'- and the 5'-end, respectively, are obtained. That method has been used, for example, by 20 Fitzpatrick *et al.* (Applied Microbiology and Biotechnology 42, 575-580 (1994)) to exclude the *recA* gene of *C. glutamicum*.

Figure 1 shows by way of example the plasmid vector pCR2.1luxRint, by means of which the *luxR* gene can be 25 disrupted or excluded.

In the gene replacement method, a mutation, such as, for example, a deletion, insertion or base substitution, is produced *in vitro* in the gene in question. The allele that is produced is in turn cloned into a vector that is not 30 replicative for *C. glutamicum*, and the latter is then transferred to the desired host of *C. glutamicum* by transformation or conjugation. After homologous recombination by means of a first cross-over occurrence effecting integration and by means of a suitable second 35 cross-over occurrence effecting an excision in the target

gene or in the target sequence, incorporation of the mutation or of the allele is achieved. That method has been used, for example, by Peters-Wendisch et al. (Microbiology 144, 915-927 (1998)) to exclude the pyc gene of *C.*

5 glutamicum by means of a deletion.

A deletion, insertion or a base substitution may thus be incorporated into the luxR gene.

In addition, it may be advantageous for the production of L-amino acids, particularly L-lysine, in addition to
10 attenuating the luxR gene, to amplify, in particular to overexpress, one or more enzymes of the biosynthesis pathway in question, of glycolysis, of the anaplerotic pathway, of the pentose phosphate cycle or of amino acid export.

15 Accordingly, for example for the production of L-lysine, one or more genes selected from the group

- the gene dapA coding for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gene eno coding for enolase (DE: 19947791.4),
- 20 • the gene zwf coding for the zwf gene product (JP-A-09224661),
- the gene pyc coding for pyruvate carboxylase (Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998))),
- the gene lysE coding for lysine export (DE-A-195 48 222)

25 may at the same time be enhanced, in particular overexpressed.

In addition, it may be advantageous for the production of amino acids, particularly L-lysine, in addition to attenuation of the luxR gene, at the same time to attenuate
30 one or more genes selected from the group

- the gene pck coding for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),
- the gene pgi coding for glucose-6-phosphate isomerase (US 09/396,478, DSM 12969),
- 5 • the gene poxB coding for pyruvate oxidase (DE:1995 1975.7, DSM 13114).

It may also be advantageous for the production of amino acids, particularly L-lysine, in addition to attenuating the luxR gene, to exclude undesired secondary reactions

10 (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

- The microorganisms produced according to the invention also
- 15 form part of the invention and can be cultivated, for the purposes of the production of L-amino acids, in particular L-lysine, continuously or discontinuously in the batch, fed batch or repeated fed batch process. A summary of known cultivation methods are [sic] described in the textbook of
- 20 Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook of Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).
- 25 The culture medium to be used must meet the requirements of the strains in question in a suitable manner. Descriptions of culture media for various microorganisms are to be found in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology
- 30 (Washington D.C., USA, 1981). There may be used as the carbon source sugars and carbohydrates, such as, for example, glucose, saccharose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for

example, soybean oil, sunflower oil, groundnut oil and coconut oil, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid. Those substances may be used individually or in the form of a mixture.

There may be used as the nitrogen source organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or in the form of a mixture.

There may be used as the phosphorus source phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts. The culture medium must also contain salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, may be used in addition to the above-mentioned substances. Suitable precursors may also be added to the culture medium. The mentioned substances may be added to the culture in the form of a single batch, or they may be fed in in a suitable manner during the cultivation.

In order to control the pH value of the culture, basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acid compounds, such as phosphoric acid or sulfuric acid, are expediently used. In order to control the development of foam, anti-foams, such as, for example, fatty acid polyglycol esters, may be used. In order to maintain the stability of plasmids, suitable substances having a selective action, such as, for example, antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or gas mixtures

containing oxygen, such as, for example, air, are introduced into the culture. The temperature of the culture is normally from 20°C to 45°C and preferably from 25°C to 40°C. The culture is continued until the maximum amount of the desired product has formed. That aim is normally achieved within a period of from 10 hours to 160 hours.

Methods of determining L-amino acids are known from the prior art. The analysis may be carried out, for example, as described in Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion-exchange chromatography with subsequent ninhydrin derivatization, or it may be carried out by reversed phase HPLC, as described in Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The following microorganisms were deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty:

- Escherichia coli strain TOP10F/pCR2.1luxRint as DSM 13619.

The process of the invention is used for the production of amino acids, in particular L-lysine, by fermentation.

The present invention is explained in greater detail below by means of Examples.

The isolation of plasmid DNA from Escherichia coli and all techniques for restriction, Klenow and alkaline phosphatase treatment were carried out according to Sambrook et al.

(Molecular Cloning. A Laboratory Manual, 1989, Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY, USA).

Methods for the transformation of Escherichia coli are also described in that handbook.

The composition of common nutrient media, such as LB or TY medium, will also be found in the handbook of Sambrook *et al.*

Example 1

- 5 Preparation of a genomic cosmid gene library from *C. glutamicum* ATCC 13032

Chromosomal DNA from *C. glutamicum* ATCC 13032 was isolated as described in Tauch *et al.* (1995, Plasmid 33:168-179) and partially cleaved with the restriction enzyme Sau3AI
10 (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, Code no. 1758250). The DNA of cosmid
15 vector SuperCos1 (Wahl *et al.* (1987), Proceedings of the National Academy of Sciences, USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vektor Kit, Code no. 251301), was cleaved with the restriction enzyme XbaI (Amersham Pharmacia,
20 Freiburg, Germany, product description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product
25 description BamHI, Code no. 27-0868-04). The cosmid DNA so treated was mixed with the treated ATCC13032 DNA, and the batch was treated with T4-DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DNA ligase, Code no. 27-0870-04). The ligation mixture was then packed in
30 phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, Code no. 200217).

For infection of *E. coli* strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575), the cells were taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. Infection and titration of the cosmid library 5 were carried out as described in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) + 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones 10 were selected.

Example 2

Isolation and sequencing of the luxR gene

The cosmid DNA of an individual colony was isolated using the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, 15 Hilden, Germany) according to the manufacturer's instructions, and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline 20 phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, Product No. 1758250). After separation by gel electrophoresis, cosmid fragments having a size in the range from 1500 to 2000 bp were isolated using the QiaExII Gel Extraction Kit (Product No. 25 20021, Qiagen, Hilden, Germany).

The DNA of sequencing vector pZero-1, obtained from Invitrogen (Groningen, Netherlands, product description Zero Background Cloning Kit, Product No. K2500-01), was cleaved with the restriction enzyme BamHI (Amersham 30 Pharmacia, Freiburg, Germany, product description BamHI, Product No. 27-0868-04). Ligation of the cosmid fragments into the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture

being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). The ligation mixture was then electroporated into E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences, U.S.A., 87:4645-4649) (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-347) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 μ g/ml Zeocin.

Plasmid preparation of the recombinant clones was carried out using the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). Sequencing was effected by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. Separation by gel electrophoresis and analysis of the sequencing reaction was carried out in a "Rotiphorese NF Acrylamid/Bisacrylamid" gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) using the "ABI Prism 377" sequencing device from PE Applied Biosystems (Weiterstadt, Germany).

The resulting crude sequence data were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZerol derivatives were assembled to a coherent contig. The computer-assisted coding region analysis was prepared using the program XNIP (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out using the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:33893402) against the non-redundant data bank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence gave an open reading

frame of 639 bp, which was designated the luxR gene. The luxR gene codes for a polypeptide of 212 amino acids.

Example 3

Preparation of an integration vector for integration
5 mutagenesis of the luxR gene

Chromosomal DNA was isolated from strain ATCC 13032 by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)). On the basis of the sequence of the luxR gene known from Example 2 for *C. glutamicum*, the following
10 oligonucleotides were selected for the polymerase chain reaction:

luxRintA:

5'GGA ATC GAC GTC ATC TTG AT 3'

luxRintB:

15 5'GCA ACC AGC TTG AGA ACT TC 3'

The primers shown were synthesised by MWG Biotech (Ebersberg, Germany), and the PCR reaction was carried out according to the standard PCR method of Innis et al. (PCR protocols. A Guide to Methods and Applications, 1990,
20 Academic Press) using Pwo polymerase from Boehringer. With the aid of the polymerase chain reaction, an internal fragment of the luxR gene having a size of 353 bp was isolated and is shown in SEQ ID No. 3.

The amplified DNA fragment was ligated into vector pCR2.1-
25 TOPO (Mead et al. (1991), Bio/Technology 9:657-663) using the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalog Number K4500-01).

E. coli strain TOP10F was then transformed with the ligation batch (Hanahan, in: DNA cloning. A Practical
30 Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA, 1985). The selection of plasmid-carrying cells was carried out by plating out the transformation batch on LB agar

(Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and was tested by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was named pCR2.1luxRint.

10 Example 4

Integration mutagenesis of the luxR gene in the lysine producer DSM 5715

Vector pCR2.1luxRint mentioned in Example 3 was electroporated in *C. glutamicum* DSM 5715 by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)). Strain DSM 5715 is an AEC-resistant lysine producer. Vector pCR2.1luxRint is unable to replicate independently in DSM 5715 and is retained in the cell only if it has integrated into the chromosome of DSM 5715. The selection of clones with pCR2.1luxRint integrated into the chromosome was effected by plating out the electroporation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) which had been supplemented with 15 mg/l kanamycin.

In order to demonstrate the integration, the luxRint fragment was labelled by the method "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) using the Dig hybridization kit from Boehringer. Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)) and cleaved with each of the restriction enzymes SalI, SacI and HindIII. The resulting fragments were separated by means of agarose gel

electrophoresis and hybridized at 68°C using the Dig hybridization kit from Boehringer. Plasmid pCR2.1luxRint mentioned in Example 3 had inserted into the chromosome of DSM 5715 within the chromosomal luxR gene. The strain was
5 designated DSM 5715::pCR2.1luxRint.

Example 5

Production of L-lysine

The *C. glutamicum* strain DSM 5715::pCR2.1luxRint obtained in Example 4 was cultivated in a nutrient medium suitable
10 for the production of L-lysine, and the L-lysine content in the culture supernatant was determined.

To that end, the strain was first incubated for 24 hours at 33°C on agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l). Starting from
15 that agar plate culture, a pre-culture was inoculated (10 ml of medium in 100 ml Erlenmeyer flasks). CgIII complete medium was used as the medium for the pre-culture.

Cg III medium

NaCl	2.5 g/l
Bacto-peptone	10 g/l
Bacto-yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH value was adjusted to
pH 7.4

Kanamycin (25 mg/l) was added thereto. The pre-culture was
20 incubated for 24 hours at 33°C at 240 rpm on a shaker. A main culture was inoculated from that pre-culture, so that the initial OD (660 nm) of the main culture was 0.1 OD. MM medium was used for the main culture.

MM medium

CSL (corn steep liquor)	5 g/l
MOPS	20 g/l
Glucose (autoclaved separately)	50 g/l
Salts:	
(NH ₄) ₂ SO ₄ [sic]	25 g/l
KH ₂ PO ₄	0.1 g/l
MgSO ₄ * 7 H ₂ O	1.0 g/l
CaCl ₂ * 2 H ₂ O	10 mg/l
FeSO ₄ * 7 H ₂ O	10 mg/l
MnSO ₄ * H ₂ O	5.0 mg/l
Biotin (sterilised by filtration)	0.3 mg/l
Thiamin * HCl (sterilised by filtration)	0.2 mg/l
Leucine (sterilised by filtration)	0.1 g/l
CaCO ₃	25 g/l

CSL, MOPS and the salt solution are adjusted to pH 7 with ammonia water and autoclaved. The sterile substrate and vitamin solutions are then added, as well as the dry
5 autoclaved CaCO₃.

Cultivation is carried out in a volume of 10 ml in a 100 ml Erlenmeyer flask with baffles. Kanamycin (25 mg/l) was added. Cultivation was carried out at 33°C and 80% humidity.

10 After 72 hours, the OD was determined at a measuring wavelength of 660 nm using a Biomek 1000 (Beckmann

Instruments GmbH, Munich). The amount of L-lysine that had formed was determined using an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion-exchange chromatography and post-column derivatization with 5 ninhydrin detection.

The result of the test is shown in Table 1.

Table 1

Strain	OD(660)	Lysine HCl g/l
DSM 5715	7.5	13.01
DSM 5715::pCR2.1luxRint	8.8	15.41

SEQUENCE PROTOCOL

<110> Degussa-Hüls AG

5 <120> Novel nucleotide sequences coding for the luxR gene

<130> 000177 BT

<140>

10 <141>

<160> 3

<170> PatentIn Ver. 2.1

15

<210> 1

<211> 1052

<212> DNA

20 <213> Corynebacterium glutamicum

<220>

<221> CDS

<222> (214)..(849)

<223> luxR gene

25

<400> 1

tgcagcattg ccggtggagc caccagaggg gtttgcggg gcgcccgttt tggcagattc 60

30 ggactcaagt gctacaggcg aggttgaact aagttctcca actgacgatg agtaaggcta 120

gactaaagta cgattcatct gctcatcgat actcttgaag gcgcattttc attcgaaacg 180

aagtgcgcca ttgggaagga cctagttcaa aca atg att cgc gtg ctg ctt gct 234

35 Met Ile Arg Val Leu Leu Ala
1 5

gat gac cac gaa atc gtg agg ctc gga ctc cga gct gtg ctg gaa agc 282

Asp Asp His Glu Ile Val Arg Leu Gly Leu Arg Ala Val Leu Glu Ser
10 15 20

40

gcc gag gac att gaa gtg gtg ggc gaa gtc tcc acc gcc gaa ggt gcg 330

Ala Glu Asp Ile Glu Val Val Gly Glu Val Ser Thr Ala Glu Gly Ala
25 30 35

45

gtg cag gca gcc caa gaa ggc gga atc gac gtc atc ttg atg gac ctc 378

Val Gln Ala Ala Gln Glu Gly Gly Ile Asp Val Ile Leu Met Asp Leu
40 45 50 55

50

cga ttc ggc ccc ggc gtc caa gga acc cag gtt tcc aca ggc gca gac 426

Arg Phe Gly Pro Gly Val Gln Gly Thr Gln Val Ser Thr Gly Ala Asp
60 65 70

55

gcc acc gca gcc atc aag cga aac atc gat aac ccg cca aaa gtc ctg 474

Ala Thr Ala Ala Ile Lys Arg Asn Ile Asp Asn Pro Pro Lys Val Leu
75 80 85

60

gtc gtg acc aac tac gac acc gac aca gac atc ctc ggc gca atc gaa 522

Val Val Thr Asn Tyr Asp Thr Asp Thr Asp Ile Leu Gly Ala Ile Glu
90 95 100

gcc ggc gca ctg ggc tac ctg ctc aaa gac gcc cca ccg agc gaa ctc 570

Ala Gly Ala Leu Gly Tyr Leu Leu Lys Asp Ala Pro Pro Ser Glu Leu
105 110 115

	ctg gca gca gta cga tcc gca gca gaa ggt gac tcc aca ctg tca ccc	618
	Leu Ala Ala Val Arg Ser Ala Ala Glu Gly Asp Ser Thr Leu Ser Pro	
5	120 125 130 135	
	atg gtt gcg aac cgc ctg atg act cgc gtg cgc acc ccc aaa acc tca	666
	Met Val Ala Asn Arg Leu Met Thr Arg Val Arg Thr Pro Lys Thr Ser	
	140 145 150	
10	ctc acc cca cgt gaa ctg gaa gtt ctc aag ctg gtt gcc ggt gga tcc	714
	Leu Thr Pro Arg Glu Leu Glu Val Leu Lys Leu Val Ala Gly Ser	
	155 160 165	
15	tcc aac cgc gac att ggc cgt atc ctc ttc ctc tca gaa gcc acg gtg	762
	Ser Asn Arg Asp Ile Gly Arg Ile Leu Phe Leu Ser Glu Ala Thr Val	
	170 175 180	
20	aaa tcc cac ctc gtg cac atc tac gac aag ctc ggc gtg cgg tca cgt	810
	Lys Ser His Leu Val His Ile Tyr Asp Lys Leu Gly Val Arg Ser Arg	
	185 190 195	
25	acc tcc gct gtc gca gcc gca cgt gag cag ggg ctg ctg tagcgggggt	859
	Thr Ser Ala Val Ala Ala Ala Arg Glu Gln Gly Leu Leu	
	200 205 210	
	tgctgcaagg ctttaggtat ccgcgccggg gttggcctac gggagcatcc cgaggcttta	919
	gcagggggcac gggctctggc ttgggctgag tcagggggcgc ggccaatgct ttccgacgcg	979
30	tgtctccacg gctttattta gtttttcaag aagtttgacg aaggtgcgta gatcctcttc	1039
	gggccagtct gaa	1052
35	<210> 2	
	<211> 212	
	<212> PRT	
	<213> Corynebacterium glutamicum	
40	<400> 2	
	Met Ile Arg Val Leu Leu Ala Asp Asp His Glu Ile Val Arg Leu Gly	
	1 5 10 15	
45	Leu Arg Ala Val Leu Glu Ser Ala Glu Asp Ile Glu Val Val Gly Glu	
	20 25 30	
	Val Ser Thr Ala Glu Gly Ala Val Gln Ala Ala Gln Glu Gly Gly Ile	
	35 40 45	
50	Asp Val Ile Leu Met Asp Leu Arg Phe Gly Pro Gly Val Gln Gly Thr	
	50 55 60	
55	Gln Val Ser Thr Gly Ala Asp Ala Thr Ala Ala Ile Lys Arg Asn Ile	
	65 70 75 80	
	Asp Asn Pro Pro Lys Val Leu Val Val Thr Asn Tyr Asp Thr Asp Thr	
	85 90 95	
60	Asp Ile Leu Gly Ala Ile Glu Ala Gly Ala Leu Gly Tyr Leu Leu Lys	
	100 105 110	
	Asp Ala Pro Pro Ser Glu Leu Leu Ala Ala Val Arg Ser Ala Ala Glu	
	115 120 125	

Gly Asp Ser Thr Leu Ser Pro Met Val Ala Asn Arg Leu Met Thr Arg
 130 135 140
 5 Val Arg Thr Pro Lys Thr Ser Leu Thr Pro Arg Glu Leu Glu Val Leu
 145 150 155 160
 Lys Leu Val Ala Gly Gly Ser Ser Asn Arg Asp Ile Gly Arg Ile Leu
 165 170 175
 10 Phe Leu Ser Glu Ala Thr Val Lys Ser His Leu Val His Ile Tyr Asp
 180 185 190
 15 Lys Leu Gly Val Arg Ser Arg Thr Ser Ala Val Ala Ala Arg Glu
 195 200 205
 Gln Gly Leu Leu
 210

20

<210> 3

<211> 353

<212> DNA

25 <213> *Corynebacterium glutamicum*

<220>

<223> luxRint

30 <400> 3

ggaatcgacg	tcattcttgat	ggacctccga	ttcggccccc	gcgtccaagg	aacccaggtt	60
tccacaggcg	cagacgccac	cgcagccatc	aagcgaaaca	tcgataaccc	gccaaaagtc	120
ctggctcgtga	ccaactacga	caccgacaca	gacatcctcg	gcgcaatcga	agccggcgca	180
ctgggctacc	tgctcaaaga	cgccccaccg	agcgaactcc	tggcagcagt	acgatccgca	240
gcagaagggtg	actccacact	gtcaccatg	ggtgcgaacc	gcctgatgac	tcgcgtgcgc	300
acccccaaaa	cctcactcac	cccacgtgaa	ctggaagttc	tcaagctggt	tgc	353

The following figures are attached:

Figure 1: Map of plasmid pCR2.1luxRint.

The abbreviations and names used have the following meanings.

KmR:	kanamycin resistance gene
EcoRI:	cleavage site of the restriction enzyme EcoRI
luxRint:	internal fragment of the luxR gene
ColE1 ori:	origin of replication of plasmid ColE1

Patent claims

1. An isolated polynucleotide from coryneform bacteria,
containing a polynucleotide sequence coding for the
luxR gene, selected from the group
 - 5 a) polynucleotide that is at least 70% identical with
a polynucleotide that codes for a polypeptide
containing the amino acid sequence of SEQ ID No. 2,
 - b) polynucleotide that codes for a polypeptide
containing an amino acid sequence that is at least
10 70% identical with the amino acid sequence of SEQ
ID No. 2,
 - c) polynucleotide that is complementary to the
polynucleotide of a) or b), and
 - 15 d) polynucleotide containing at least 15 consecutive
nucleotides of the polynucleotide sequence of a),
b) or c),

the polypeptide preferably exhibiting the activity of
the transcription activator LuxR.
2. A polynucleotide as claimed in claim 1, wherein the
20 polynucleotide is a DNA, preferably a recombinant DNA,
that is replicatable in coryneform bacteria.
3. A polynucleotide as claimed in claim 1, wherein the
polynucleotide is an RNA.
4. A replicatable DNA as claimed in claim 2, containing
 - 25 (i) the nucleotide sequence shown in SEQ ID No. 1,
or
 - (ii) at least one sequence that corresponds to
sequence (i) within the region of the degeneracy
of the genetic code, or

- (iii) at least one sequence that hybridizes with the sequences that are complementary to sequence (i) or (ii), and optionally
- (iv) sense mutations in (i) that are neutral in terms of function.
- 5
6. A coryneform bacteria in which the luxR gene is attenuated, preferably excluded.
7. A process for the preparation of L-amino acids, in particular L-lysine, which process comprises carrying out the following steps:
- 10
- a) fermenting the bacteria that produce the desired L-amino acid, in which bacteria at least the luxR gene is attenuated,
- b) concentrating the desired product in the medium or in the cells of the bacteria, and
- 15
- c) isolating the L-amino acid.
8. A process as claimed in claim 7, wherein there are used bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced.
- 20
9. A process as claimed in claim 7, wherein there are used bacteria in which at least some of the metabolic pathways that reduce formation of the desired L-amino acid are excluded.
- 25
10. A process as claimed in claim 7, wherein expression of the polynucleotide(s) coding for the luxR gene is diminished, in particular excluded.
11. A process as claimed in claim 7, wherein the regulatory properties of the polypeptide for which the polynucleotide luxR codes are diminished.
- 30

12. A process as claimed in claim 7, wherein for the production of L-amino acids, particularly L-lysine, bacteria are fermented in which at the same time one or more genes selected from the group
- 5 12.1 the gene *dapA* coding for dihydrodipicolinate synthase,
- 12.2 the gene *eno* coding for enolase,
- 12.3 the gene *zwf* coding for the *zwf* gene product,
- 12.4 the gene *pyc* coding for pyruvate carboxylase,
- 10 12.5 the gene *lysE* coding for lysine export,
- are enhanced, in particular overexpressed.
13. A process as claimed in claim 7, wherein at the same time one or more genes selected from the group:
- 15 13.1 the gene *pck* coding for phosphoenol pyruvate carboxykinase,
- 13.2 the gene *pgi* coding for glucose-6-phosphate isomerase,
- 13.3 the gene *poxB* coding for pyruvate oxidase are attenuated.
- 20 14. A process as claimed in one or more of the preceding claims, wherein microorganisms of the species *Corynebacterium glutamicum* are used.
15. A method of finding RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes,
- 25 that code for the transcription activator LuxR or have a high degree of similarity with the sequence of the *luxR* gene, which method comprises using the polynucleotide sequences as claimed in claims 1 to 4 as hybridization probes.

Novel nucleotide sequences coding for the luxR gene**Abstract**

Isolated polynucleotide containing a polynucleotide sequence selected from the group

- 5 a) polynucleotide that is at least 70% identical with a polynucleotide that codes for a polypeptide containing the amino acid sequence of SEQ ID No. 2,
 - b) polynucleotide that codes for a polypeptide containing an amino acid sequence that is at least 70% identical
10 with the amino acid sequence of SEQ ID No. 2,
 - c) polynucleotide that is complementary to the polynucleotide of a) or b), and
 - d) polynucleotide containing at least 15 consecutive
15 nucleotides of the polynucleotide sequence of a), b) or c),
- and a process for the production of L-amino acids by fermentation using coryneform bacteria in which at least the luxR gene is present in attenuated form, and the use of the polynucleotide sequences as hybridization probes.

Figure 1: Plasmid map of pCR2.1luxRint

